

1645
PATENT #10
1/15/02

Attorney Docket No.: 8117-14

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Patent application of
Graham McCreath et al.

Group Art Unit: 1645

Serial No.: 09/814,371

Filed: March 22, 2001

Examiner:

RECEIVED

For: Purification Of Fibrinogen From Fluids
By Precipitation And Hydrophobic
Chromatography

JAN 09 2002

TECH CENTER 1600/2900

SUBMISSION OF CERTIFIED PRIORITY DOCUMENTS UNDER 35 U.S.C. § 119Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith are certified copies of Great Britain patent application Nos. 9820847.3 filed September 24, 1998, 9820848.1 filed September 24, 1998 and 9820845.7 filed September 24, 1998 in the name of PPL Therapeutics (Scotland) Limited, the foreign priority of which is claimed under 35 U.S. C. § 119.

CERTIFICATE OF MAILING
UNDER 37 C.F.R. 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date indicated below, with sufficient postage, as first class mail, in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231.

BY Theresa M. KellyDATE: Dec. 20, 2001


THIS PAGE BLANK (USPTO)

Submission of the certified copies of the above-stated priority documents satisfies all of the requirements of 35 U.S.C. § 119. The right of foreign priority should therefore be accorded to the present U.S. application.

No fee is believed due. However, if a fee is due, please charge or credit any amount paid in excess to deposit account No. 50-0573.

Respectfully submitted

GRAHAM MCCREATH, et al.

BY 
DANIEL A. MONACO
Registration No. 30,480
DRINKER, BIDDLE & REATH LLP.
One Logan Square
18th and Cherry Streets
Philadelphia, PA 19103-6996
Tel. (215) 988-3312
Fax. (215) 988-2757
Attorney for the Applicants

THIS PAGE BLANK (USPTO)



INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

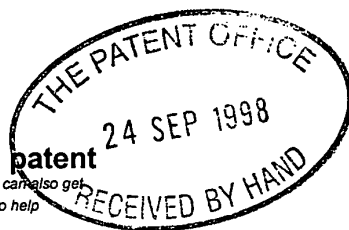
Signed

Stephen Hordley

Dated

6 December 2001

THIS PAGE BLANK (USPTO)



25SEP98 E392623-3 000056
P01/7700 25.00 - 9820847.3

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1. Your reference

KVC/P21183GB

2. Patent application number
(The Patent Office will fill in this part)

9820847.3

3. Full name, address and postcode of the or of each patent applicant (underline all surnames)

PPL Therapeutics (Scotland) Limited
Roslin
Midlothian
Edinburgh EH25 9PP

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

GB

6719858002

4. Title of the invention

METHOD

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent
(including the postcode)

KILBURN & STRODE
20 RED LION STREET
LONDON
WC1R 4PJ

Patents ADP number (if you know it)

125001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or each of these earlier applications and (if you know it) the or each application number

Country

Priority application number
(if you know it)

Date of filing
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application number

Date of filing
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

YES

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or

c) any named applicant is a corporate body.
See note (d))

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form : 0
Description : 27
Claim(s) : 3
Abstract : 0
Drawing(s) : 4

10. If you are also filing any of the following, state how many against each item.

Priority documents : -
Translations of priority documents : -
Statement of inventorship and right to grant of a patent (Patents Form 7/77) : -
Request for preliminary examination and search (Patents Form 9/77) : -
Request for substantive examination (Patents form 10/77) : -
Any other documents (please specify) : -

11. I/We request the grant of a patent on the basis of this application.

Signature Date
Kilburn & Strobe *Kilburn & Strobe* 24 September 1998

12. Name and daytime telephone number of person to contact in the United Kingdom
Kristina V. J. Cornish
Tel: 0171-539 4200

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.*
b) *Write your answers in capital letters using black ink or you may type them.*
c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*
d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*
e) *Once you have filled in the form you must remember to sign and date it.*
f) *For details of the fee and ways to pay please contact the Patent Office.*

Method

This disclosure is concerned generally with protein purification and specifically with the purification of human fibrinogen from the body fluid of transgenic non-human animals having a defined A α chain integrity.

Fibrinogen, the main structural protein in the blood responsible for the formation of clots exists as a dimer of three polypeptide chains; the A α (66.5kD), B β (52kD) and γ (46.5kD) are linked through 29 disulphide bonds.^b The addition of asparagine-linked carbohydrates to the B β and γ chains results in a molecule with a molecular weight of 340kD. Fibrinogen has a trinodal structure, a central nodule, termed the E domain, contains the amino-termini of all 6 chains including the fibrinopeptides (Fp) while the two distal nodules termed D domains contain the Carboxy-termini of the A α , B β and γ chains. Fibrinogen is proteolytically cleaved at the amino terminus of the A α and B β chains releasing fibrinopeptides A and B (FpA & FpB) and converted to fibrin monomer by thrombin, a serine protease that is converted from its inactive form by Factor Xa. The resultant fibrin monomers non-covalently assemble into protofibrils by DE contacts on neighboring fibrin molecules. This imposes a half staggered overlap mode of building the fibrin polymer chain. Contacts are also established lengthwise between adjacent D domains (DD contacts) leading to lateral aggregation. Another serine protease, Factor XIII is proteolytically cleaved by thrombin in the presence of Ca²⁺ into an activated form. This activated Factor XIII (Factor XIIIa) catalyses crosslinking of the polymerised fibrin by creating isopeptide bonds between lysine and glutamine side chains. The first glutamyl-lysyl bonds to form are on the C-terminal of the γ chains producing D-D crosslinks. Subsequently, multiple crosslinks form between adjacent A α chains, the process of crosslinking imparts on the clot both biological stability (resistance to fibrinolysis) and mechanical stability [Sienbenlist and Mosesson, Progressive Cross-Linking of Fibrin

γ chains Increases Resistance to Fibrinolysis, Journal of Biological Chemistry, 269: 28414-2841, 1994].

5 The coagulation process can readily be engineered into a self sustained adhesive system *in vitro* by having the fibrinogen and Factor XIII as one component and thrombin and Ca^{2+} as the second component which catalysis the polymerisation process. These adhesion systems, know in the art as "Fibrin Sealents" or "Fibrin Tissue Adhesives" have found numerous application in surgical procedures and as delivery devices for a range of pharmaceutically active compounds [Sierra, Fibrin Sealent Adhesive Systems: A Review of Their Chemistry, material Properties and 10 Clinical Applications, Journal of Biomaterials Applications, 7:309-352, 1993].

It has been estimated that the annual US clinical need for fibrin sealents is greatly in excess of the 300Kg/year that can be harvested using the current cryoprecipitation 15 methods used by plasma fractionaters. Alternative sources of fibrinogen, by far the major component in fibrin sealent, have therefore been explored with recombinant sources being favored [Butler et al., Current Progress in the Production of Recombinant Human Fibrinogen in the Milk of Transgenic animals, Thrombosis and Haemostasis, 78: 537-542, 1997]. It has been shown that mammals are capable of 20 producing transgenic human fibrinogen at levels of up to 5.0g/L in their milk making this a commercially viable method for the production of human fibrinogen [Prunkard et al., High-level expression of recombinant human fibrinogen in the milk of transgenic mice, Nature Biotechnology, 14:867-871, 1996; Cottingham et al., Human fibrinogen from the milk of transgenic sheep. In: Tissue Sealents: Current Practice, Future Uses. Cambridge Institute, Newton Upper Falls, MA, March 30 25 April 2 1996 (abstract)].

Differences have been identified between recombinant human fibrinogen and fibrinogen which has been purified from human plasma. Fibrinogen which has been purified from human plasma has two alternately spliced gamma chains (γ and γ'). In contrast, recombinant human fibrinogen only has the major form γ . Further, the glycosylation of the beta and gamma chains (there is no N-linked glycosylation of the alpha chain) of recombinant human fibrinogen differs slightly from that on plasma derived fibrinogen, but is similar to the glycosylation found on other proteins expressed in the milk of transgenic animals. In addition, the Ser3 of the alpha chain of recombinant human fibrinogen is more highly phosphorylated than Ser3 of the alpha chain of plasma derived fibrinogen, although the difference in phosphorylation does not result in functional differences. Also, there are detectable differences in heterogeneity caused by C-terminal proteolysis of a number of highly protease-sensitive sites on the alpha chain. Differences of a similar magnitude are also observed between plasma-derived fibrinogen from different sources.

15

Human plasma fibrinogen appears heterogeneous by SDS-PAGE and other methods for separating proteins based on size. A high molecular weight fraction (HMW Fibrinogen, Fibrinogen 1 or F1) with a molecular weight of 340,000 daltons contributes approximately 50-70% of total fibrinogen antigen. Low molecular weight fibrinogen (LMW Fibrinogen, Fibrinogen 2 or F2) with a molecular weight of approximately 300,000 daltons contributes 20-40%. The residual amount, designated as low molecular weight' fibrinogen (LMW' Fibrinogen, Fibrinogen 3, F3 or Fragment X) has a molecular weight of approximately 280,000 daltons. It has been shown that the major differences in these fibrinogen molecules results from proteolytic damage to the carboxy terminus region of the A α chains (A α C-terminal region) resulting in differing lengths of A α chain C-terminus. Fibrinogen, purified from cryoprecipitate by the use of precipitation techniques has been shown to have partially digested A α chain [Stroetmann, U. S Patent 4,427,650] Although it was

20
25

first thought that plasmin or plasmin-like enzymes were responsible for degradation of F1 fibrinogen to F2 and F3 sub-families [Lipinska et al., Fibrinogen Heterogeneity in Human Plasma: Electrophoretic demonstration and characterization of two major fibrinogen components, *Journal of Laboratory & Clinical Chemistry*, 5 84: 509-516, 1974] it is apparent that plasmin itself is probably not responsible for the direct proteolysis of F1 to F2 fibrinogen [Dempfle et al., Fibrinogen Heterogeneity in Homozygous Plasminogen Deficiency Type 1: Further evidence that plasmin is not involved in formation of LMW and LMW'- Fibrinogen, *Thrombosis and Haemostasis*, 77:879-883, 1997]. It has been suggested that F2 10 fibrinogen may actually be a group of degradation products produced by several enzymes including matrix metalloproteases [Nakashima et al., Human Fibrinogen Heterogeneity: the COOH - terminal residues of defective A α chains of fibrinogen II, *Blood Coagulation and Fibrinolysis*, 3:361-370, 1992]. Recombinant fibrinogen expressed in CHO cells has also been shown to be heterogeneous comprising of F1 15 fibrinogen and a smaller F2 - like sub fraction that is also lacking the C-terminal region of the A α chain illustrating that the recombinant fibrinogen is also susceptible to proteolysis [Gorkun et al., The conversion of fibrinogen to fibrin: Recombinant fibrinogen typifies plasma fibrinogen, *Blood* 89:4407-4414]. Similarly, recombinant human fibrinogen, produced in yeast, has also been shown to possess an F2-like 20 fraction having partially degraded A α chains [Roy et al., Secretion of Biologically Active Fibrinogen by Yeast, *Journal of Biological Chemistry*, 270: 23761-23767, 1995], demonstrating that A α chain damage may be expected for a range of expression hosts. As well as the major F2 and F3 fragments, there exist a range of smaller fragments generated from fibrinogen termed Fibrinogen Degradation 25 Products (FDPs). F3 is also often referred to as a FDP. These FDPs (Fragment Y, D and E) are well characterized and can be found in small amounts in human plasma.

Differences in the rate of clot formation, the structure of the final clot and the mechanical properties of the final clot have been observed by various investigators for each of the major fibrinogen fragments. Also, the presence of FDPs, and their influence on the clotting progress has been investigated. Gorkan et al., [Role of the α C domains of fibrin in clot formation, *Biochemistry* 33: 6986-6997, 1994] established that F1 fibrinogen is 95% clottable while F2 fibrinogen is 92% clottable. While total clottability of these two fractions appears similar, a distinct difference in clotting time i.e. onset of visible clot formation following the action of thrombin, was observed with the F2 fibrinogen exhibiting a greater lag time before clot formation. This has also been observed previously (e.g. Holm et al., 1985, Purification and Characterization of 3 Fibrinogens with different molecular weights obtained from Normal Human Plasma, *Thrombosis & Haemostasis*, 37: 165-176) where F1 fibrinogen was observed to have a clotting time of 14s compared to 20s for F2 and 25s for F3. Evidence therefore suggests that the extent of proteolysis of the $A\alpha$ c-terminus influences fibrin polymerization. The 3-dimensional structure of the clot is also influenced by the degree of degradation of the α C regions of fibrinogen. Clots formed from F2 and F3 fibrinogen exhibit a low degree of protofibril branching with increased porosity. It has been postulated that partially degraded fibrinogens are more prone to lateral aggregation of protofibrils. This leads to the formation of thicker fibers resulting in coarser clots as observed by light scattering experiments. Further evidence for the importance of the $A\alpha$ chain C-terminus in clot formation arises from the fact that clots formed from Fibrinogen Milano III, a naturally occurring variant with truncated $A\alpha$ chains exhibits a reduced degree of protofibril branching [Furlan et al., Binding of calcium ions and their effect on clotting of Fibrinogen Milano II, a variant with truncated $A\alpha$ chains, *Blood Coagulation and Fibrinolysis*, 7: 331-335, 1995]. Differences in mechanical properties of clots formed with different fibrinogen species has also been observed where clots formed from F2 and F3 appear less resistant to mechanical disturbances.

Thromboelastography (TEG) reveals that clots made from F1 fibrinogen are more elastic than clots formed from F2 fibrinogen [Hasegawa and Sasaki, Location of the binding site "b" for lateral polymerisation of fibrin, *Thrombosis Research*, 57: 183-195, 1990]. Elasticity is a preferred property for fibrin sealants whose use may include application in joint or tendon surgery.

The C-terminal regions of the A α chains also serve other purposes distinct from clot formation. They enclose crosslinking sites for the transglutaminase, Factor XIIIa where FXIIIa catalyses the formation of isopeptide bond between adjacent fibrinogen molecules thereby adding strength and stability to the clot. Crosslinking also increases the clots resistance to proteolysis and is responsible for localizing other molecules involved in the clotting process to the surface of the clot most notably α 2-antiplasmin, which is covalently crosslinked into the A α chains by Factor XIIIa further enhancing the stability of the clot to proteolytic degradation [Rudchenko et al., Comparative, Structural and Functional Features of the Human α C domain and the Isolated α C Fragment, *Journal of Biological Chemistry*, 271: 2523-2530, 1996]. Fibronectin, Thrombospondin and von Willibrands Factor are also crosslinked into this region. The A α C-regions are also important for enhancing the activation of plasminogen by tPA on the clot surface therefore leading to effective fibrinolysis [Matsuka et al., Factor XIIIa catalysed crosslinking of Recombinant α C Fragments of Human Fibrinogen, *Biochemistry*, 35: 5810-5816, 1996]. It has also been postulated that the A α C-terminus of fibrinogen encloses specific recognition sites for platelet receptors located in residues A α 572 through A α 574 [Hawiger, Adhesive ends of fibrinogen and its adhesive peptides: The end of a saga, *Seminars in Haematology*, 32:99-109, 1995]. Platelet aggregation is essential for haemostasis and therefore it may be expected that fibrinogen molecules having degraded A α chains would be less capable of aggregating platelets.

The importance of $\text{A}\alpha$ C-terminal regions to fibrinogen properties has inspired the development of techniques whereby fibrinogen molecules having varying degrees of $\text{A}\alpha$ chain proteolysis can be separated for study. Various methods have been described for the separation of the major fibrinogen sub families and FDPs. For example precipitation techniques have been used to separate F1 and F2 from purified fibrinogen [Sasaki and Kito, Simplified determination of fibrinogen sub-fractions by glycine precipitation, *Thrombosis and Haemostasis* 42: 440-443, 1979]. Holm et al., have described a method for the separation of purified plasma fibrinogen into F1, F2 & F3 subfamilies by using a series of precipitations with ammonium sulphate. F3, fragments Y, D and E have been separated based on size using size exclusion chromatography [Morder and Raphael Shulman, High molecular weight derivatives of human fibrinogen produced by plasmin, *Journal of Biological Chemistry*, 244:2120-2124, 1969]. These authors also demonstrated that F3 fibrinogen and FDPs Y, D and E actually possess anticoagulant activity and are inhibitory to clot formation; a non-desirable feature of a molecule used to prepare a surgical adhesive. Most attention has been paid to the terminal degradation products D and E which have been separated using anion exchange chromatography [Kemp et al., Plasmic degradation of fibrinogen: the preparation of a low molecular weight derivative of fragment D, *Thrombosis and Haemostasis*, 3:553-564, 1973], cation exchange chromatography [Rutjven Vermeer et al., A novel method for the purification of rat and human fibrin(ogen) degradation products, *Hoppe-Seyler's Z. Physiological Chemistry*, 360:633-637] Lysine-sepharose chromatography [Rupp et al., Fractionation of plasmic fibrinogen digest on Lysine agarose. Isolation of two fragments D, fragment E and simultaneous removal of plasmin, *Thrombosis Research*, 27:117-121, 1982] and Zinc chelated affinity chromatography [Structural features of fibrinogen associated with binding to chelated zinc, Scully and Kakkar, *Biochim et Biophys. Acta.*, 700:130-133, 1982]. In none of the above methods has

the simultaneous separation of fibrinogen into sub-fractions F1, F2 & F3 and FDPs Y, D & E been described using a single technique.

Milk is well known to contain a number of serine proteases; of these, the alkaline protease plasmin, which occurs in milk together with its inactive zymogen plasminogen, is the most significant protease contributing to proteolytic activity. Plasmin(ogen) concentration varies with health status of the animal e.g. mastitic animals exhibit increased proteolytic activity. Also influencing the proteolytic activity of milk is stage in lactation i.e. late lactation is associated with higher concentrations of plasmin [Politis and Ng Kwai Hang, Environmental Factors Affecting Plasmin Activity in Milk, Journal of Dairy Science, 72:1713-1718, 1989]. In milk, plasmin(ogen) is associated predominantly with the casein micelles, although it can also be found to a lesser extent in whey [Politis et al., Distribution of Plasminogen and Plasmin in Fractions of Bovine Milk, Journal of dairy Science, 75:1402-1410, 1992].

Plasmin is the serine protease that is predominantly responsible for the dissolution of fibrin clots *in vivo* and its presence is essential for haemostasis. However, as discussed previously, while the participation of plasmin in A α chain degradation of F1 to F2 and F3 is still under debate, it is very probable that any fibrinogen degradation product in milk will be as a result of the action of milk proteases. Therefore, the presence of plasmin or other proteases in milk can be detrimental to the quality of fibrinogen that is produced by the lactating transgenic animal if steps are not taken to minimize their effect. Of equal importance is the removal of any fibrinogen degradation products that may result from the action of plasmin or other milk proteases. The use of protease inhibitors to minimize proteolysis is well established in the art and usually involves the addition of a cocktail of inhibitors of varying specificity.

From the above discussion, it is clear that the incorporation of fibrinogen degradation products (F3 fibrinogen, fragments Y, D & E) and even F2 fibrinogen into preparations whose end-use would be either as a hemostasis or sealing agents is not desirable. Techniques which can be incorporated into a purification of fibrinogen from the milk of transgenic animals which reduce fibrinogen degradation products enabling fibrinogen with a defined A α chain integrity to be produced for varying applications would be of considerable use.

Accordingly, the present invention provides a method for obtaining fibrinogen from a fluid, the method comprising:

- (a) contacting the fluid with a hydrophobic interaction chromatography resin under conditions where the fibrinogen binds to the resin; and
- (b) removing the bound protein by means of elution.

As used herein the term "fibrinogen" refers to the main structural protein responsible for the formation of clots and includes the whole glycoprotein form of fibrinogen as well as other related fibrinogen species including truncated fibrinogen, amino acid sequence variants (muteins or polymorphic variants) of fibrinogen, a fibrinogen species which comprises additional residues, and any naturally occurring allelic variants of fibrinogen.

Hydrophobic Interaction Chromatography (HIC) resins are known in the art and include resins such as Butyl SepharoseTM. (Amersham Pharmacia Biotechnology), Phenyl Sepharose (low and high substitution), Octyl Sepharose and Alkyl Sepharose

(all of Amersham Pharmacia Biotechnology; other sources of HICs include Biosepra, France; E. Merck, Germany; BioRad USA).

5 The fluid-containing fibrinogen may be any. In particular, it is one or more animal body-fluids such as milk, blood plasma or urine. It is, in particular a fluid-containing fibrinogen which is or has been derived from a body fluid of an animal (such as one of those described above) and/or a fluid which has been used to solvate the fibrinogen, for example following a previous method step such as part-purification by precipitation.

10

Conditions under which the fibrinogen-containing fluid is contacted with the hydrophobic interaction chromatography resin to enable fibrinogen to bind to the resin include the presence of any "structure forming" salt (solution), such as ammonium sulphate, sodium sulphate and other salts as described in: Melander and
15 Horuath, Salt Effects on Hydrophobic Interactions in Precipitation and Chromatography: An Interpretation of the Lyotropic Series, Archives of Biochemistry and Biophysics, 183:200-215, 1977 and Srinivason and Ruckenstein, Role of Physical Forces in Hydrophobic Interaction Chromatography, Separation and Purification Methods, 9: 267-370, 1980. Removal of the bound protein is by means
20 of standard elution techniques known in the art. Such elution can be carried out by decreasing the concentration of the structure forming salt, such as decreasing the concentration of ammonium sulphate in the eluting buffer. Elution may be by gradient elution or more preferably, by a series of steps to predetermine and thus define the fibrinogen that is eluted from the column in terms of its sub-fraction ratios
25 and hence its A α chain integrity.

Preferably, the method of the first aspect of the invention includes a step of washing the resin, to remove unbound components, between steps (a) and (b). Washing the

resin is usually carried out with a washing buffer which has the same concentration of salt in it that was used for loading. A higher concentration of salt in the washing buffer is possible, but not preferred.

5 Any animal body fluid can be used according to the method of the present invention. Preferred body fluids include milk, blood plasma or urine. Clearly, the natural production of fibrinogen in some body fluids, such as plasma, can provide an animal body fluid from which naturally occurring fibrinogen can be isolated. However, the present invention is preferably in relation to the isolation of transgenic fibrinogen as
10 a result of transgenic manipulation of an animal. This accordingly, allows for the isolation of fibrinogen from animal body fluids which do not naturally contain fibrinogen, such as milk and urine. The present invention is useful in for the production of fibrinogen *per se* or fibrinogen which has been altered in some way to facilitate transgenic expression, such as by fusion to other proteins.

15 As used herein, "milk" is understood to be the fluid secreted from the mammary glands of animals and includes the whole milk, skimmed milk or indeed any milk fraction. The term "blood plasma" includes whole blood plasma and any fraction thereof. The term "urine" also refers to whole urine, or fractions thereof, in
20 particular concentrated urine.

The method according to the first aspect of the invention preferably achieves at least one of the following:

- 25
- (a) increases the purity of the fibrinogen
 - (b) resolves the fibrinogen into its fractions
 - (c) enables isolation of higher integrity fibrinogen A α chain.

Since the present invention takes advantage of genetic manipulation of animals in order to obtain proteins from transgenic sources ("transgenic fibrinogen"), the source of the fibrinogen can be carefully selected. Preferably, the fibrinogen is human, bovine or ovine derived. For medical purposes, it is preferred to employ proteins native to the intended patient. Thus human transgenic fibrinogen is preferred. Where the fibrinogen is recombinantly encoded, so that fibrinogen from a species other than the species in which it is being expressed, the glycosylation pattern may be different from the glycosylation pattern of the naturally occurring fibrinogen. A transgenic animal closer in biological taxonomy to the source of the transgenic fibrinogen may thus be preferred.

Clearly, any animal which produces a body fluid which may be used according to the first aspect of the invention is contemplated. Preferably, animals which can be genetically manipulated to produce transgenic fibrinogen in their milk, are preferred. In this respect, animals which lactate and produce suitable milk include sheep, goat, cow, camel, rabbit, water buffalo, pig or horse. These animals are also useful for the production of other body fluids according to the invention. Transgenic animals for the production of a transgenic protein according to the present invention, do not include transgenic humans.

In order to achieve the maximum result from the method according to the first aspect of the invention, it may be preferable to at least partially purify the fibrinogen from the animal body-fluid. Such a purification will depend on the body fluid from which the fibrinogen is derived and the nature of potential contaminants present. The fibrinogen is preferably purified to a level of from 20 through 40% before undergoing the method according to the first aspect of the invention. Any pre-purification method can be used, for example those known in the art, e.g. precipitation of fibrinogen as described in PCT WO 95/22249. The fact that the

fibrinogen may already be part purified before application of the first aspect of the invention, does not detract from the fact the fibrinogen may have been originally produced in the body fluid of an animal.

- 5 A second aspect of the present invention provides purified fibrinogen which has high integrity of the fibrinogen A α chain. The selection of such fibrinogen is enabled by the method of the first aspect of the invention.

- 10 A third aspect of the invention provides for purified fibrinogen obtainable by a method according to the first aspect of the invention.

A fourth aspect of the invention provides fibrinogen 1 (F1), fibrinogen 2 (F2), or a combination thereof, which has high integrity of A α chains.

- 15 The fifth aspect of the invention provides fibrinogen 1 (F1), fibrinogen 2 (F2), or a combination thereof, obtainable by a method according to the first aspect of the invention.

- 20 The fibrinogen 1 and/or fibrinogen 2, according to the fourth and fifth aspects of the invention are particularly preferred for use in fibrinogen adhesives or sealents as described hereinbefore and hereinafter.

- 25 The second, third, fourth and fifth aspects of the invention preferably produce fibrinogen which is substantially free from viral contamination. Such fibrinogen can more easily be produced from non-blood derived products such as those from milk or urine.

The fibrinogen according to the present invention may be in any suitable or convenient state, such as in a lyophilised or soluble state.

5 All preferred features of the first aspect of the invention also apply to the second, third, fourth and fifth aspects of the invention.

A sixth aspect of the invention provides for the use of HIC in one or more of the following:

- 10 (a) increasing the purity of fibrinogen
(b) resolving fibrinogen into its fractions
(c) selecting of fibrinogen with high integrity of A α chains

from fibrinogen in a fluid, preferably a body fluid from an animal.

15

The use of HIC in the sixth aspect of the invention is preferably in combination with a salt solution as described according to the first aspect of the invention. Relevant preferred features of aspects one to five, also apply to the sixth. The use of the HIC in all relevant aspects of the invention includes a batch format or a column format.

20 In batch format, the liquid may be contacted with the HIC resin in a well stirred tank. Separation of the HIC resin from the liquid may then be facilitated by sedimentation or be centrifugally assisted. In column format, which is preferred, the liquid is preferably pumped through a column into which HIC resin has already been added. Column formats are preferred as they result in greater adsorption efficiency.

25 This column format could be regarded as either a "Packed" or "Fixed" bed format. Further, "Expanded bed" or "fluidized bed" contactors may also be applicable.

A seventh aspect of the invention provides a fibrin adhesive or sealent containing fibrinogen according to any of the second, third, fourth or fifth aspects of the invention.

5 As used herein, the term "fibrin adhesive" or "fibrin sealent" describes a substance containing fibrinogen which is capable of forming a biodegradable adhesive or seal by the formation of polymerised fibrin. Such adhesive/sealant systems are alternatively called "fibrin tissue adhesives" or "fibrin tissue glues". The adhesive or seal may act as, *inter alia* a hemostatic agent, a barrier to fluid, a space-filling
10 matrix or a drug-delivery agent. Particular use may be found in neurosurgery, ophthalmic, orthopedic or cardiothoracic surgery, skin grafting and various other types of surgery.

Other than fibrinogen, the fibrin adhesive or sealent may contain substances which
15 encourage the formation of the fibrin adhesive/seal, such as thrombin, Ca^{++} and Factor XIII (and/or Factor XIIIa). While it is recognised that thrombin is the preferred enzyme with which to incorporate into any system whereby the formation of a fibrin clot is desired, it is appreciated that there are other enzymes capable of proteolytically cleaving fibrinogen resulting in the formation of a fibrin clot. An
20 example of this would be the snake venom enzyme Batroxobin [Weisel and Cederholm-Williams, Fibrinogen and Fibrin: Characterization, Processing and Applications, *Handbook of Biodegradable Polymers* (Series: Drug targeting and Delivery) 7:347-365, 1997]. Other components such as albumin, fibronectin, solubilisers, bulking agents and/or suitable carriers or diluents may also be included
25 if desired.

One advantage of fibrin sealent as a biodegradable polymer is that there are natural mechanisms in the body for the efficient removal of clots and thus the fibrin sealent

may be a temporary plug for hemostasis or wound healing. Various proteolytic enzymes and cells can dissolve fibrin depending on the circumstances, but the most specific mechanism involves the fibrinolytic system. The dissolution of fibrin clots under physiological conditions involves the binding of circulating plasminogen to fibrin, and the activation of plasminogen to the active protease, plasmin, by plasminogen activators which may also be, bound to fibrin. Plasmin then cleaves fibrin at specific sites.

Depending on the situation, it may be advantageous to let the natural process of fibrin breakdown take place after applying a fibrin adhesive or sealent to a site. Indeed, this breakdown may be encouraged, for example, by the inclusion of plasminogen. Alternatively, in some situations it may be advantageous to delay the process by including antifibrinolytic compounds which can, for example, block the conversion of plasminogen to plasmin or directly bind to the active site of plasmin to inhibit fibrinolysis. Such antifibrinolytics include α_2 -macroglobulin, which is a primary physiological inhibitor of plasmin; aprotinin; α_2 -antiplasmin; and ϵ -amino caproic acid.

The fibrin/sealant may comprise two components, one component containing fibrinogen and Factor XIII (and/or Factor XIIIa) and the other component containing thrombin and Ca^{++} . Other substances as described above may be included in one or both of the components if desired.

All the preferred features of the first to sixth aspects of the invention, also apply to the seventh.

An eighth aspect of the invention provides a kit for a fibrin adhesive or sealant. The kit may comprise fibrinogen according to the second, third, fourth or fifth aspect of

the invention and instructions for use or, may comprise fibrinogen according to the second, third, fourth or fifth aspect of the invention in combination with (but not necessarily mixed with) one or more of: Factor XIII, Factor XIIIa, thrombin or Ca^{++} . Furthermore, the kit may comprise two components: fibrinogen with (but
5 **not** necessarily mixed with) Factor XIII (and/or Factor XIIIa) and thrombin with (but not necessarily mixed with) Ca^{++} .

The components of any fibrinogen sealent according to the present invention, including the kit forms, may be used separately, simultaneously or sequentially.

10

All preferred features of aspects one to seven of the invention, also apply to the eighth.

A ninth aspect of the invention provides a method for producing a fibrin adhesive or
15 sealent comprising mixing together fibrinogen according to any one of the second to fifth aspects of the invention and thrombin.

Factor XIII (and/or Factor XIIIa) and Ca^{2+} may also be mixed with the fibrinogen and thrombin in this aspect of the invention.

20

The method may involve squirting or spraying the components simultaneously or sequentially to the repair site with a syringe or a related device. The mixing may result from two syringes held together along their barrels and at the plungers with the two components mixed either after exiting the needles or in the hub just prior to
25 exiting. Other devices may be used to produce an aerosol or to spray in a variable pattern, depending on the application.

Preferred features of aspects one to eight, also apply to the ninth aspect.

A tenth aspect of the invention provides fibrinogen, according to the second, third, fourth or fifth aspect of the invention, for use in medicine. Preferably the fibrinogen is used in human medicine, However, it may also be used in veterinary medicine
5 such as for horses, pigs, sheep, cattle, mice and rats as well as for domestic pets such as dogs and cats.

While the main use of fibrinogen is thought to be for the preparation of adhesive or sealing agents as hereinbefore described, fibrinogen has other applications in the
10 field of medicine, for example as a coating for polymeric articles as disclosed in US Patent No 5,272,074. A particular use of lyophilised fibrinogen of the present invention is within or part of a gauze or bandage (preferably made from polylactic acid compounds used in surgical stitches). Such a wound dressing can be supplied
15 (also incorporating the other components required for the formation of a clot (described above), optionally in a package or kit form, for application direct to the skin or to an internal organ.

Preferred features of aspects one to nine of the invention, also apply to the tenth.

20 An eleventh aspect of the invention provides a method of surgery or therapy comprising placing fibrinogen according to any one of the second to fifth aspects of the invention on or within an animal or a body part of an animal. The fibrinogen may be mixed with one or more of: thrombin, Factor XIII, Factor XIIIa or Ca^{++} separately, sequentially or simultaneously with the fibrinogen. The fibrinogen may
25 thus be in the form of a sealant according to the seventh aspect of the invention. The fibrinogen may be applied by squirting using a syringe or a related device. It may be applied very precisely in a localised area or broadly over a wide area to any biological tissue.

Preferred features of aspects one to ten also apply to the eleventh.

- 5 A twelfth aspect of this invention provides the use of fibrinogen according to the second, third, fourth or fifth aspect of the invention in the manufacture of a fibrin adhesive or sealant.

Preferred features of aspects one to eleven, also apply to the twelfth.

- 10 In this invention, purification of fibrinogen is achieved by the use of Hydrophobic Interaction Chromatography (HIC) which is carried out in such a way that enables not only the separation of milk proteins, leading to a substantially pure product, but also the simultaneous fractionation of fibrinogen into F1, F2 and degradation products. In general, fibrinogen, preferably partially purified by precipitation, is
15 contacted with a HIC resin (e.g. Butyl Sepharose™) under conditions where the fibrinogen is retained (e.g. 0.2-0.8M, preferably 0.3 to 0.6M, ammonium sulphate). The resin is then washed, either in batch fashion by centrifugation or by inclusion in a chromatography column. Elution of bound material is facilitated by decreasing the concentration of salt (e.g. ammonium sulphate in decreasing concentration 0.5 to
20 0M) in the mobile phase so that resolution of fibrinogen from non-fibrinogen components is achieved. By careful selection of salt concentration, the fibrinogen is not only separated from the majority of milk components but can also be fractionated into subfamilies. Elution can either be carried out using a decreasing gradient whereby the slope of the gradient determines the resolution or, more conveniently,
25 by use of a series of decreasing steps of concentration. The use of HIC enables the fibrinogen product to be defined with respect to its A α C-terminal region

The plasminogen activation system in milk has been a focus of interest for a number of years. It is generally accepted that milk contains the primary enzymes responsible for fibrinolysis *in vivo* e.g. plasminogen activator (both tissue type, tPA and urokinase type, uPA), plasminogen and plasmin. The action of proteolysis is often observed during storage of milk or milk products where casein appears to be the milk protein most susceptible to degradation. It was soon illustrated that in milk, plasminogen activators, plasminogen and plasmin were associated mainly with the casein micelles and not in the whey (or serum) phase. The mechanism by which these molecules associate with casein has not been categorically determined but it is probable that as these molecules contain Kringle domains (structured polypeptide chains with an affinity for basic amino acids) these domains probably mediate their interaction with casein.

It is realized that proteolysis of the human protein may occur within the mammary gland or udder of the lactating transgenic animal. The incubation period of the transgenic protein in the mammary gland or udder can be approximated to the time period between milking of the animal. Therefore it is apparent that increasing the frequency of milking minimizes this time period. However, increasing the frequency of milking to above 3 or 4 milkings per day not only creates a measure of discontinuity for the animal but involves a cost addition to Dairy expenses. It is accepted therefore that a measure of degradation of the human fibrinogen will occur. As discussed in the Prior Art, the presence of fibrinogen degradation products in a fibrin tissue adhesive compromises the usefulness of the product and therefore any degradation products must be removed. This invention discloses how the inventors have discovered an extremely efficient way of achieving this which also allows the ratio of F1 and F2 fibrinogen in the final product to be selected and defined.

Techniques for the separation of plasma fibrinogen into its various sub-fractions, as described in the Prior Art, generally fall into two categories. Those which rely on the differential solubility of subfractions in high concentration of salts (e.g. ammonium sulphate and glycine), often referred to as selective precipitation techniques [Holm et al., Purification and Characterisation of 3 Fibrinogens with different molecular weights obtained from normal human plasma, Thrombosis Research, 37: 165-176, 1985], and those which take advantage of the fact that degradation products have a different molecular size and can therefore be separated using size exclusion chromatography.

10

The two categories of techniques described above are quite contrasting in their ability and ease of use, at industrially enabling scales, for subfractionating fibrinogen. While precipitation techniques are relatively easy to operate and scale, their inherent mode of separation does not allow for the extremely high levels of resolution that would be required to ensure that the fibrinogen produced could be accurately defined with respect to its F1 : F2 ratio and hence A α . chain integrity. Indeed, advocates of this technique at the laboratory scale often report contamination of subfractions with each other [Lipinska et al., Fibrinogen Heterogeneity in Human Plasma: Electrophoretic demonstration and characterization of two major fibrinogen components, Journal of Laboratory & Clinical Chemistry, 84: 509-516, 1974] and low yields.

20

In contrast to techniques based on differential solubility, size exclusion chromatography can potentially result in very good resolution of fibrinogen sub-fractions in high yield. The main drawbacks of this technique are expense and scale. Although F1 & F2 fibrinogen and F2 & F3 differ by some 35-40Kdal, the size of the molecule itself (340Kdal) is near the limit of the fractionation range of most size exclusion matrices. This results in poor resolution if expensive resins are not used.

25

Another limitation is scale, as SEC is not a chromatographic technique favored at process scale when subtle separations have to be carried out. Also, SEC is usually a very expensive technique as only a small fraction of a column volume of material could be loaded while maintaining resolution.

5

Hydrophobic Interaction Chromatography (HIC) is a separations technique which exploits the binding of proteins to mildly hydrophobic resins in the presence of low concentrations of salts which expose hydrophobic patches on the surface of proteins. In the presence of these so-called "structure forming" salts, selective interactions can be initiated between different proteins and the matrix. The technique is most often used to discriminate between different proteins in a heterogeneous mixture. The inventors have discovered that not only is HIC a very good fractionation technique for the recovery of fibrinogen from a partially purified extract, it is also a surprisingly powerful technique for resolving fibrinogen sub-fractions i.e. F1, F2, F3 (Fragment X), Fragment Y and Fragments D & E.

15

Transgenic human fibrinogen, partially purified from milk is bound to HIC resins (e.g. but not exclusively Butyl Sepharose[®] 4FF, Amersham Pharmacia Biotechnology) in the presence of ammonium sulphate or other "structure forming" salt at a concentration enabling fibrinogen to bind e.g. a range 0.2-1.0M (preferably 0.3-0.6M) is used. By decreasing the concentration of ammonium sulphate in the irrigation buffer, the bound material elutes from the column in the order milk components (0.485-0.37M ammonium sulphate), F1 fibrinogen (0.37-0.2M ammonium sulphate), F2 fibrinogen (0.2-0.14M ammonium sulphate) and F3 fibrinogen and degradation products (0.10-0.0M ammonium sulphate). The range of concentrations of ammonium sulphate over which the bound components elute is determined, in part, by the operating conditions and those skilled in the art would be able to adjust either the temperature or the pH or both to change the concentrations

20

25

of ammonium sulphate over which the fractions elute. Using this technique it is possible by means of gradient elution or more preferably by a series of steps to predetermine and thus define the fibrinogen that it is eluted from the column in terms of its F1 to F2 ratio and hence its A α chain integrity.

5

This text refers to the accompanying figures of which:

Figure 1 is an chromatogram illustrating the various fractions generated from the HIC column in example 1.

10

Figure 2 is SDS-PAGE of transgenic human fibrinogen elution from an HIC column in example 1.

15

Figure 3 is SDS-PAGE of transgenic human fibrinogen elution from an HIC column in example 2.

Figures 4 is chromatogram illustrating the various fractions generated from the HIC column in example 2.

20

Figures 5, 6, 7 and 8 are RP-HPLC chromatograms for fibrinogen and fibrinogen fractions eluted from the HIC column using conditions outlined in examples 1 and 2.

The following examples illustrate, but do not limit, the present invention.

25

Example 1

Transgenic fibrinogen was, partially purified from the milk of a transgenic ewe by precipitation. 2ml was made to 0.485M ammonium sulphate by the addition of

1.45M ammonium sulphate in 5mM citrate, pH 7.5 (1ml). After mixing, the solution was pumped onto a HiTrap Butyl Sepharose® 4FF column (previously equilibrated with 0.485M ammonium sulphate, in 25mM citrate buffer, pH 7.5) at 0.1ml/min. The column was washed with 2 column volumes of 0.485M ammonium sulphate in 5mM citrate, pH 7.5 after which elution was carried out in 3 steps 1) 0.40M ammonium sulphate in 5mM citrate, pH 8.0, 2) 0.15M ammonium sulphate in 5mM citrate buffer, pH 8.0, 3) 5mM citrate, pH 8.0. The chromatogram presented below as Figure 1 shows that 4 major peaks were obtained from this experiment. The first peak represents material that does not bind to the column under these adsorption conditions and is mainly sheep milk proteins. The second peak represents material that did bind to the column and was eluted with 0.40M ammonium sulphate. The third peak represents fibrinogen and fractions taken across this are shown on a SDS-PAGE as Figure 2. The clear distinction between F1 (High molecular weight fibrinogen) and F2 (Low molecular weight fibrinogen) and the resolution obtained on the chromatography can be clearly seen (Lanes 1-5). Lane 6 represents the pooled peak while lanes 7 & 8 represent peak 4 from the chromatogram which can be seen to be F3 (Fragment X) fibrinogen. Thus it is evident that by changing the concentration of ammonium sulphate used for elution it is possible to define eluted fibrinogen with respect to its α chain integrity.

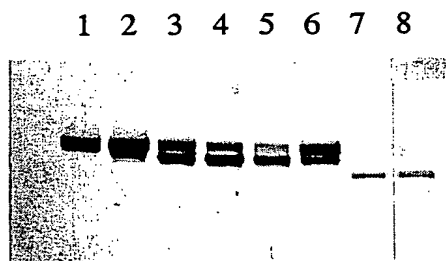


Figure 2. SDS-PAGE of transgenic human fibrinogen

elution from a Butyl Sepharose 4
FF column.

5 Example 2

In another example which illustrates the scale-up potential of this technique, a procedure equivalent to example 1 above was scaled up by a factor of 400. Thus 0.9g (790ml) of transgenic human fibrinogen was partially purified by precipitation.
10 It was then made to 0.5M ammonium sulphate by the addition of 790ml of 1M ammonium sulphate in 5mM citrate buffer pH 7.5. This material was loaded onto a column 5cm x 21cm (400ml) of Butyl Sepharose 4FF at a flow rate of 20ml/min. After loading, the column was washed with 400ml of 0.5M ammonium sulphate in 5mM citrate buffer, pH 7.5. Bound material was eluted from the column by
15 irrigation with three buffers 1) 0.4M ammonium sulphate in 5mM citrate, pH 7.5, 800ml 2) 0.15M ammonium sulphate in 5mM citrate buffer, pH 7.5, 800ml, and 3) 5mM citrate, pH 7.5, 800ml.

20 1 2 3 4 5 6 7 8 9

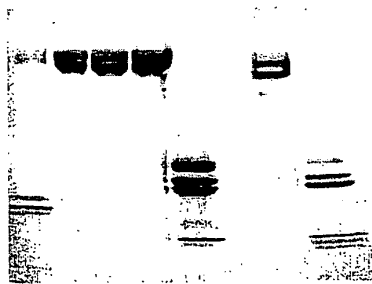


Figure 3. SDS-PAGE of
transgenic human fibrinogen

elution from a Butyl Sepharose 4
FF column.

5 The SDS-PAGE and chromatogram shown as Figures 3 and 4 respectively, show
results for this experiment. As can be seen from the SDS-PAGE, F1:F2 fibrinogen
was eluted from the column by 0.15M ammonium sulphate (Lanes 3-4, Figure 3)
while F3 fibrinogen was eluted using a step change to 5mM citrate, pH 7.5
containing no ammonium sulphate (Lane 8, Figure 3). Reducing SDS-PAGE is a
convenient way of determining $\text{A}\alpha$ chain integrity as loss of $\text{A}\alpha$ C-terminal regions
10 results in a decrease in the $\text{A}\alpha$ chain molecular weight. This decrease is readily
qualitatively assessed. In Figure 3, Lane 6 shows a reduced F1:F2 fibrinogen with
10mM dithiothreitol as the reducing agent. When this is compared to F3 fibrinogen
(Lane 8), the loss of $\text{A}\alpha$ chain is clearly seen.

15 Quantitative information on $\text{A}\alpha$ chain integrity can be obtained by the use of
Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) on reduced
fibrinogen according to Raut et al., [Ultra-rapid preparation of milligram quantities
of the purified polypeptide chains of human fibrinogen, Journal of Chromatography
B, 660:390-394, 1994] which allows for integration of peak areas. Figure 5 shows a
20 RP-HPLC chromatogram for purified F1 fibrinogen; the three fibrinogen chains
elute from the column in the order $\text{A}\alpha$, $\text{B}\beta$ and γ respectively; as can be seen, there
exists a single peak for each chain. Integration of the $\text{A}\alpha$ chain results in a peak area
which is used as a standard against which fibrinogen with degraded $\text{A}\alpha$ chains can
be normalized. In Figure 6, a RP-HPLC chromatogram, run under identical
25 conditions to that in Figure 5, is shown for F2 fibrinogen where it is evident that the
 $\text{A}\alpha$ peak has been separated into two peaks, the former being intact $\text{A}\alpha$ chain and
the latter being $\text{A}\alpha$ chain being proteolytically cleaved at the C-terminus. Using on-
line integration it can be calculated that the $\text{A}\alpha$ chain exist as 73% intact, the

remaining 27% being degraded A α chain. In Figure 7 a RP-HPLC chromatogram is shown for F3 fibrinogen. In this chromatogram it is evident that amount of degraded A α greatly outweighs the amount of non-degraded A α chain as is illustrated by the much reduced non-degraded A α chain peak. It can be calculated that degraded A α chain represent 62% of total A α chain present.

It is evident therefore that using the technique of RP-HPLC, as an analytical tool following Hydrophobic Interaction Chromatography, allows conditions for the Hydrophobic Interaction Chromatography to be selected to prepare fibrinogen with a defined A α chain integrity. An example of this is given in Figure 8 which represents elution from the Butyl Sepharose 4 FF column using conditions outlined in Example 2 above. From the chromatogram in Figure 8 it is evident that mainly F1 fibrinogen is selected as the A α chain is 87% intact.

CLAIMS

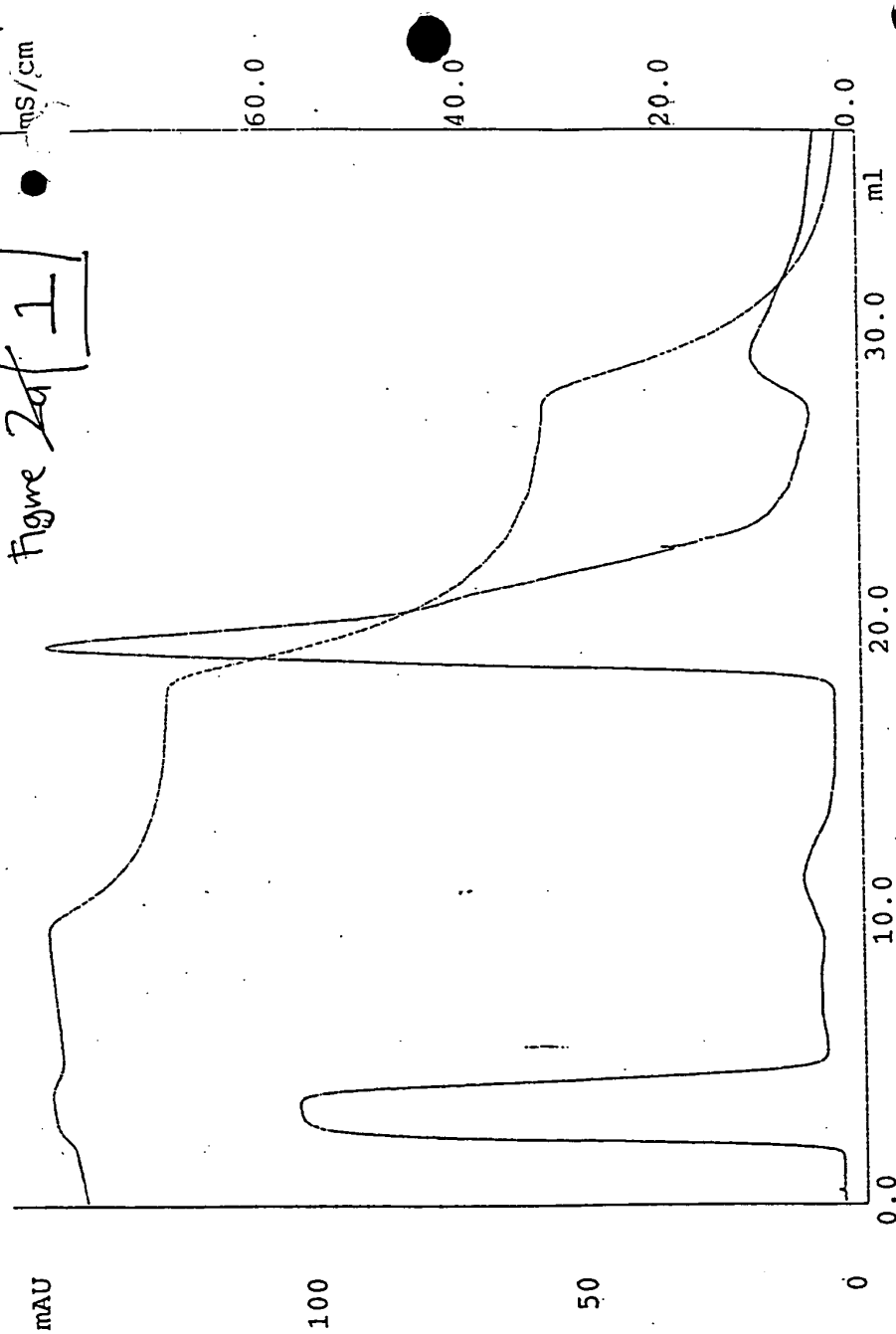
1. A method for obtaining fibrinogen from a fluid, the method comprising;
 - 5 (a) contacting the fluid with a Hydrophobic Interaction Chromatography resin under conditions where the fibrinogen binds to the resin; and
 - (b) removing the bound protein by means of elution.
- 10 2. A method, as claimed in claim 1, further including a step of washing the resin to remove unbound components, between steps (a) and (b).
3. A method, as claimed in claim 1 or claim 2, wherein the fluid is milk, blood plasma or urine.
- 15 4. A method, as claimed in any one of claims 1 to 3, wherein the protein is a transgenic protein.
5. A method, as claimed in any one of claims 1 to 3, wherein the conditions in
 - 20 (a) are an ammonium sulphate concentration of 0.15 to 1.5M preferably 0.15 to 1.0M.
6. A method, as claimed in any one of claims 1 to 5, wherein the method achieves at least one of the following:
 - 25 (a) increases the purity of the fibrinogen
 - (b) resolves the fibrinogen into its fractions
 - (c) enables selection of fibrinogen having integral A α chains.

7. A method, as claimed in any one of claims 1 to 6, wherein the fibrinogen is human or bovine derived.
- 5 8. A method, as claimed in any one of claims 1 to 7, wherein the animal from which the fluid is obtained is a sheep, cow, goat, rabbit, camel, water-buffalo, pig or horse.
9. A method, as claimed in any one of claims 1 to 8, wherein the fibrinogen-containing fluid has been at least partially purified from the body fluid of an animal.
- 10 10. Purified fibrinogen which has increased integrity of the fibrinogen A α chain.
11. Purified fibrinogen, obtainable by any one of claims 1 to 9.
- 15 12. Fibrinogen 1, fibrinogen 2, or a combination thereof, which has increased integrity of A α chains.
13. Fibrinogen 1, fibrinogen 2, or a combination thereof, obtainable by a method
- 20 described in any one of claims 1 to 9.
14. The use of HIC in one or more of the following:
- (a) increasing the purity of fibrinogen
- 25 (b) resolving fibrinogen into its fractions
- (c) selecting fibrinogen having integral A α chains.
15. The use of HIC, as claimed in claim 14, with a salt solution.

16. A fibrin adhesive or sealent comprising fibrinogen, as claimed in any one of claims 10 to 13.
- 5 17. A kit for a fibrin adhesive or sealent comprising fibrinogen, as claimed in any one of claims 10 to 13 together with thrombin as one component and Factor XIII and/or Factor XIIIa and Ca^{++} as a second component.
- 10 18. A method for producing a fibrin adhesive or sealent, as claimed in claim 16, comprising mixing together fibrinogen, Factor XIII, thrombin and Ca^{++} .
19. Fibrinogen, as claimed in any one of claims 10 to 13, for use in medicine.
- 15 20. A method of surgery or therapy comprising placing a fibrinogen adhesive or sealent, as claimed in claim 16, on or within an animal or a body part of an animal.
21. A method as claimed in claim 20, wherein the animal is a human.
- 20 22. The use of fibrinogen, as claimed in any one of claims 10 to 13, in the manufacture of a fibrin adhesive or sealent.
23. A method for obtaining fibrinogen from a fluid, substantially as hereinbefore described with reference to any one of the examples.
- 25 24. Fibrinogen, substantially as hereinbefore described with reference to any one of the examples.

18Mara:1_UV1_280nm — 18Mara:1_Con

Figure 2a [1]



THIS PAGE BLANK (USPTO)

Sample: HIC one
Acquired: 27-MAY-97 11:06
Channel: UV absorbance
Method: C:\MAX\DATA1\GCCRZAT2
Comments: Butyl Sepharose 4FF of Q₁ fraction - batch 1

Filename: Test1
Operator:

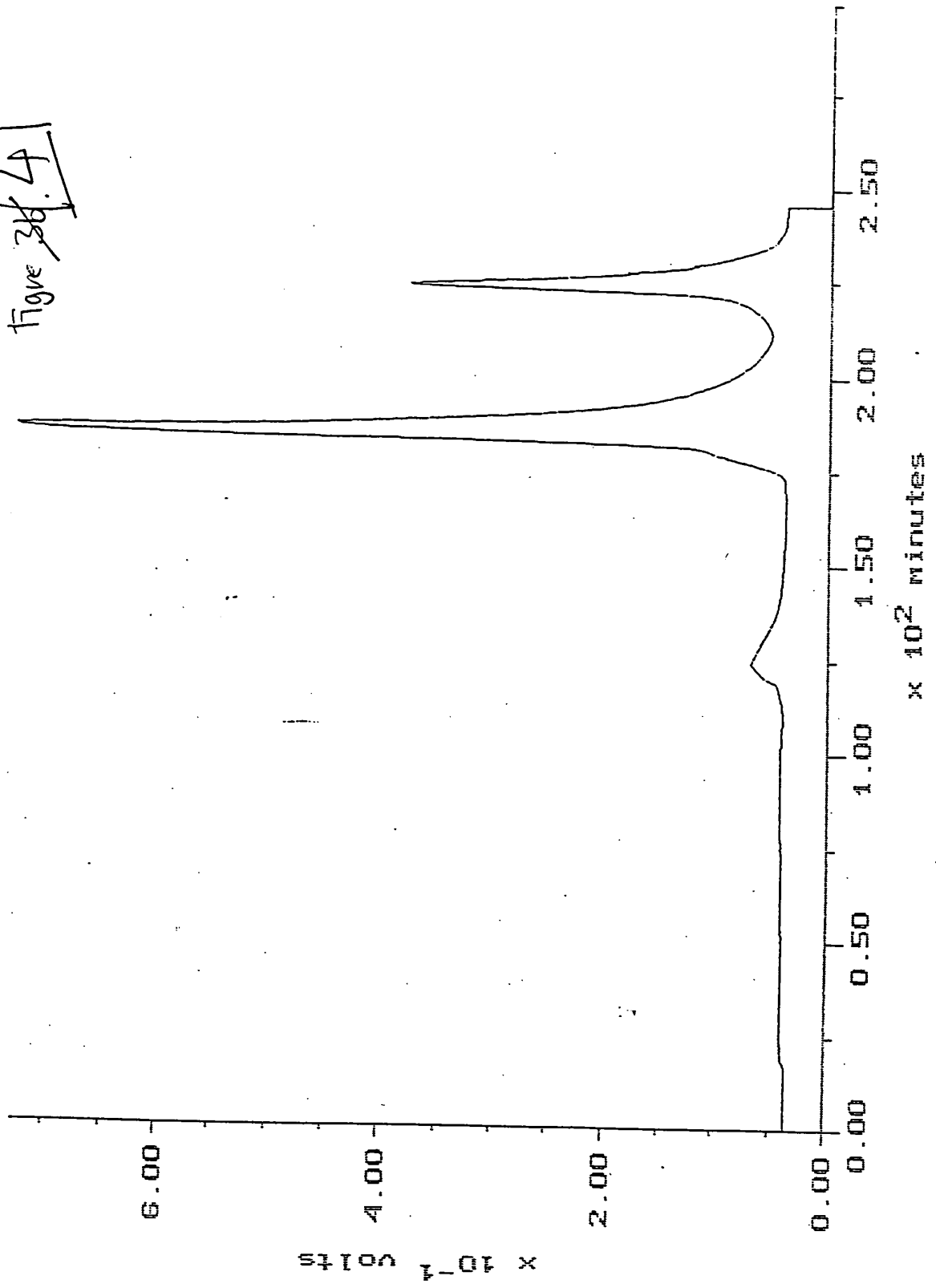
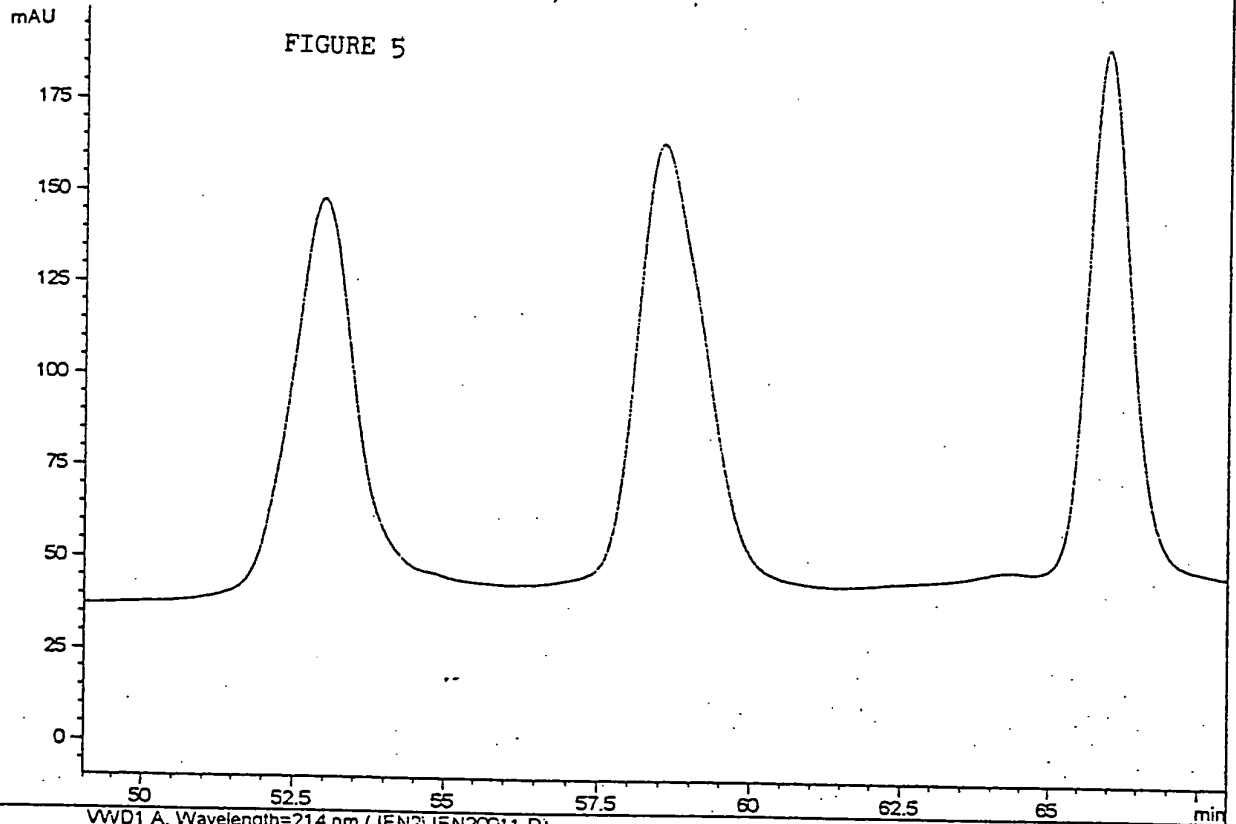


Figure 36.4

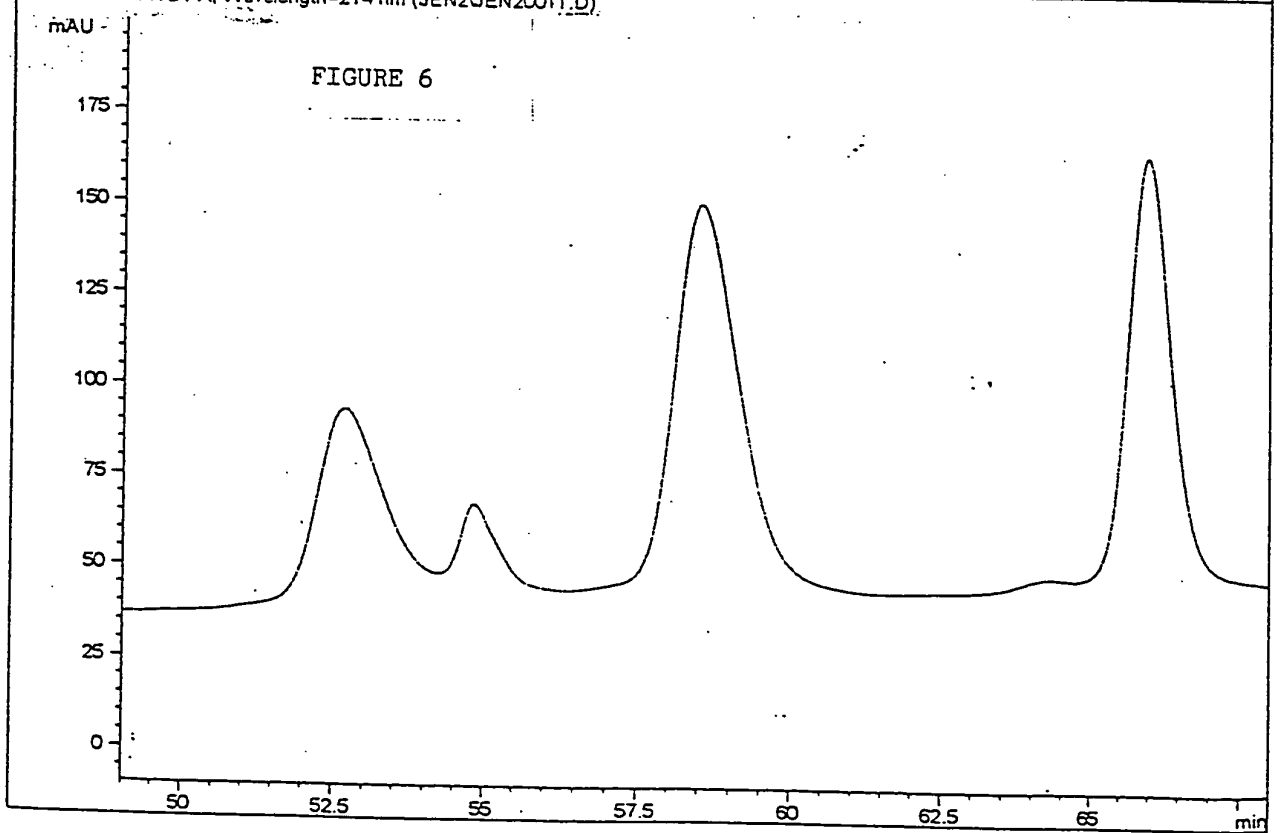
THIS PAGE BLANK (USPTO)

Current Chromatogram(s)

VWD1 A, Wavelength=214 nm (JEN2JEN20010.D)

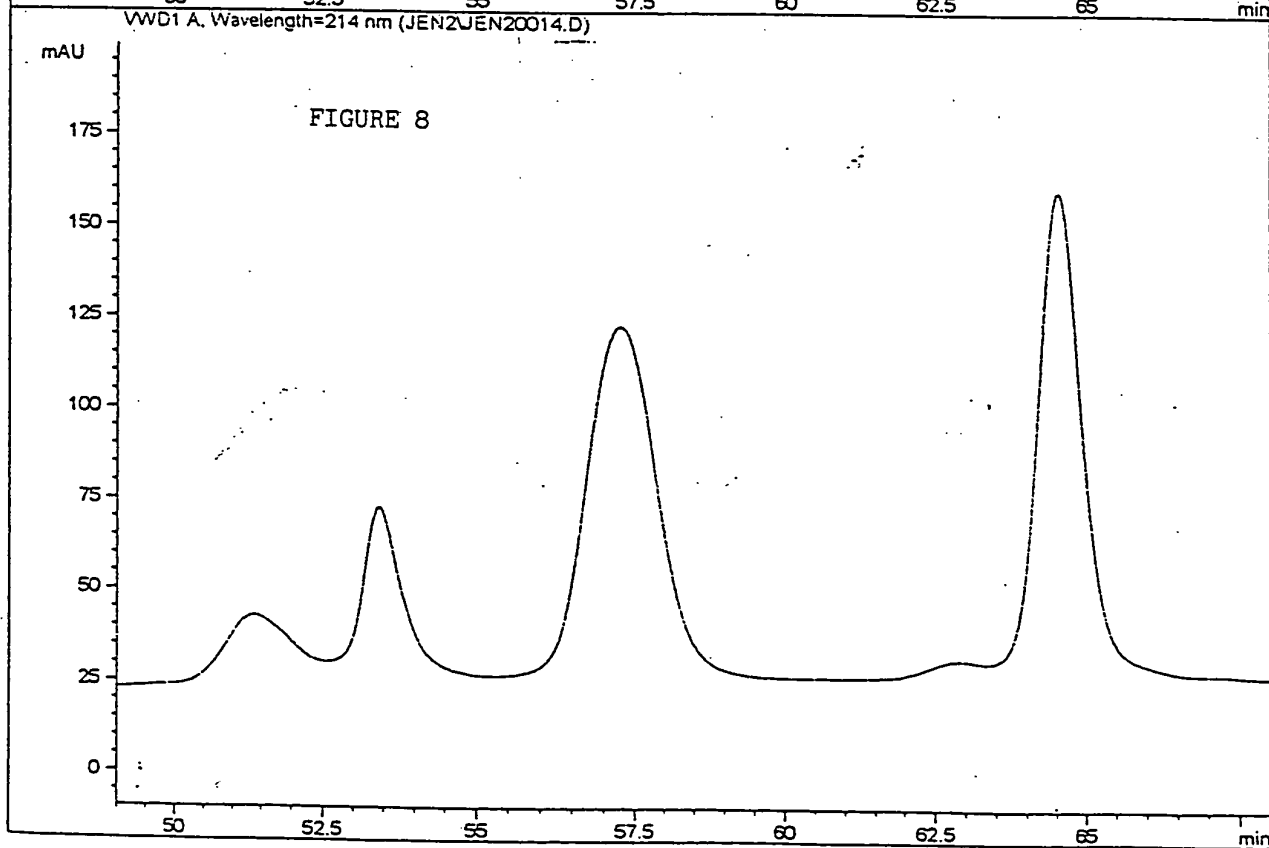
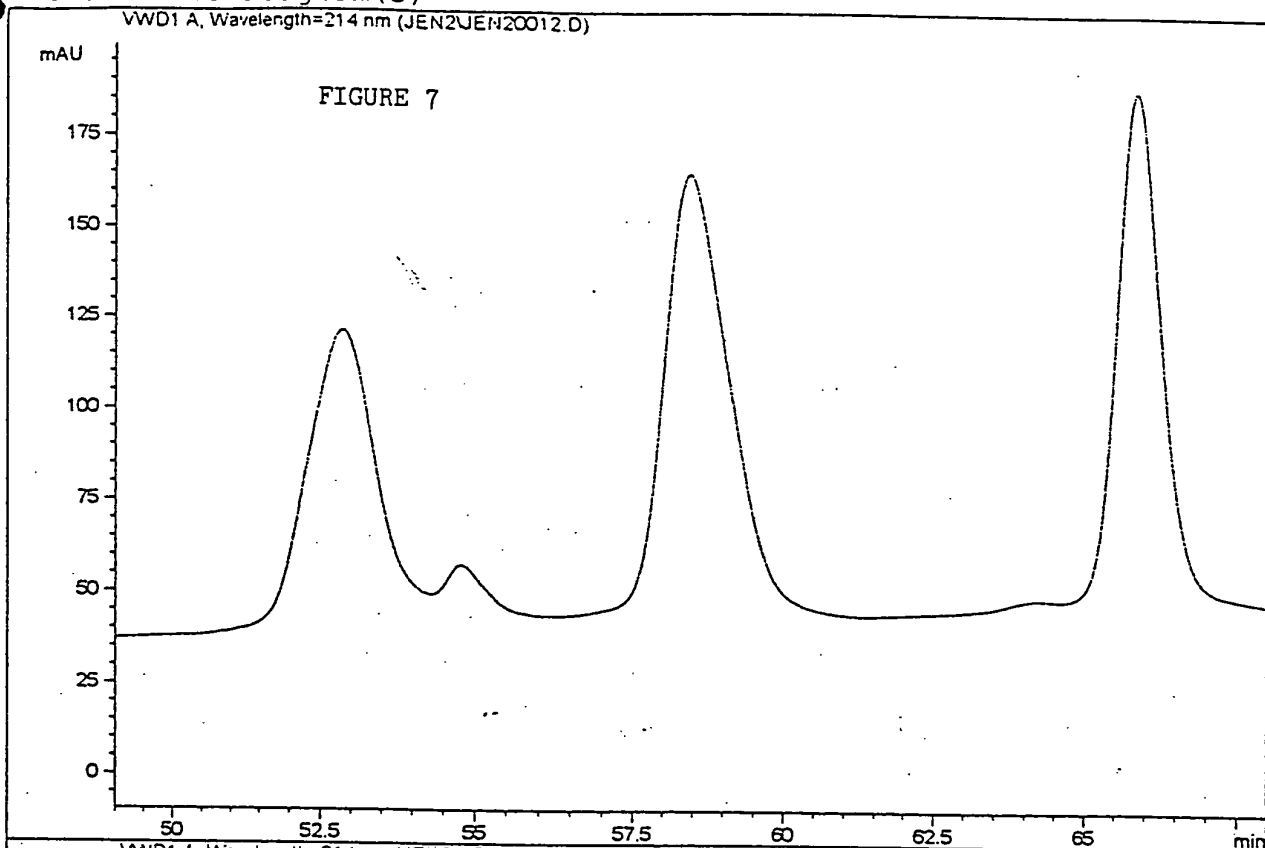


VWD1 A, Wavelength=214 nm (JEN2JEN20011.D)



THIS PAGE BLANK (USPTO)

Current Chromatogram(s)



THIS PAGE BLANK (USPTO)